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A2ML1 and otitis media: novel variants, differential expression and relevant pathways

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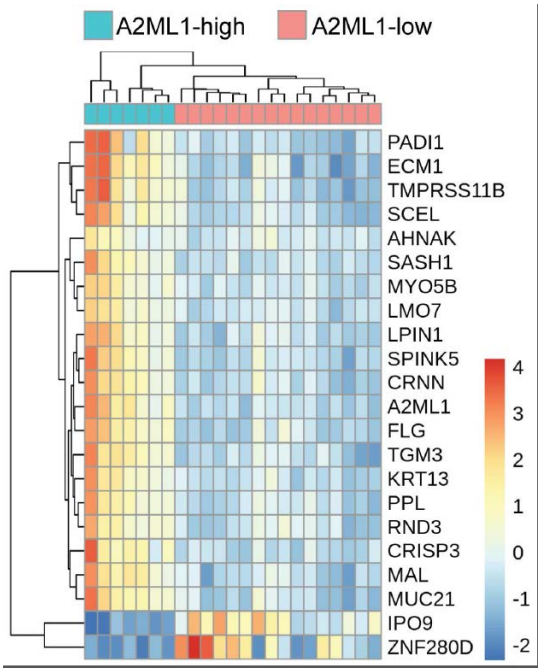
Abstract

A genetic basis for otitis media is established, however the role of rare variants in disease etiology is largely unknown. Previously a duplication variant within *A2ML1* was identified as a significant risk factor for otitis media in an indigenous Filipino population and in US children. In this report exome and Sanger sequencing was performed using

DNA samples from the indigenous Filipino population, Filipino cochlear implantees, US probands, and Finnish and Pakistani families with otitis media. Sixteen novel, damaging *A2ML1* variants identified in otitis media patients were rare or low-frequency in population-matched controls. In the indigenous population, both gingivitis and *A2ML1* variants including the known duplication variant and the novel splice variant c.4061+1G>C were independently associated with otitis media. Sequencing of salivary RNA samples from indigenous Filipinos demonstrated lower *A2ML1* expression according to carriage of *A2ML1* variants. Sequencing of additional salivary RNA samples from US patients with otitis media revealed differentially expressed genes that are highly correlated with *A2ML1* expression levels. In particular *RND3* is upregulated in both *A2ML1* variant carriers and high-*A2ML1*-expressors. These findings support a role for *A2ML1* in keratinocyte differentiation within the middle ear as part of otitis media pathology and the potential application of ROCK inhibition in otitis media.

Graphical Abstract

In this report novel rare or low-frequency variants were identified in multiple populations, including a novel splice variant c.4061+1G>C that, together with gingivitis and a known A2ML1 duplication variant, confer increased susceptibility to otitis media in an indigenous Filipino population. RNA-sequencing revealed that pathogenic A2ML1 variants decrease A2ML1 transcript levels, while changes in A2ML1 levels in otitis media patients result in differential expression of multiple genes that are known to be involved in mucosal, epithelial or infectious traits. Our findings support a role for A2ML1 in keratinocyte differentiation within the middle ear as part of otitis media pathology and the potential application of ROCK inhibition in otitis media.



Keywords: A2ML1, alpha-2-macroglobulin-like-1, exome sequencing, otitis media, RNA-sequencing

Introduction

Otitis media is a very common and costly disease in young children that can cause hearing loss and further lead to speech and reading difficulties (le Clerq et al. 2017; Khavarghazalani et al. 2016; Carroll et al. 2017; Cai & McPherson, 2017). Known risk factors for otitis media include young age, lack of breastfeeding, allergies, upper respiratory infection, second-hand smoke, low social status, day care attendance, multiple siblings and family history (Brennan-Jones et al. 2015; Zhang et al. 2014). In the US, otitis media incidence in children remains high at 6%, 23% and 46% at ages 3, 6 and 9 months, respectively (Chonmaitree et al. 2016). In pediatric and adult emergency departments, 2.2% and 6.8% of visits are due to ear complaints and nearly two-thirds of these complaints are diagnosed as otitis media (Kozin et al. 2015). Annual health care expenditures due to office visits, antibiotics, and surgeries for US children <30 months old is estimated to cost $5 billion (Casey & Pichichero, 2014).

The persistence of high incidence of otitis media in children despite maximization of public health interventions point to other risk factors including immune weaknesses and genetic predisposition. Heritability of otitis media ranges from 22-74% depending on otitis media type and cohort (Casselbrant et al. 1999; Hafrén et al. 2012). The identification of genetic risk factors and disease-related pathways is one area of otitis media study for which efficient tools are available but discovery remains very limited compared to other common complex, inflammatory, immune, or infectious disorders.

While the most current catalog of genome-wide association studies (GWAS) lists >3,500 studies, only five studies (<0.15%) using common single nucleotide polymorphisms (SNP) identified significant loci for OM susceptibility, namely: intergenic rs10497394 on 2q31.1 (Allen et al. 2013); rs16974263 in the 19q13.2 region which is intronic to *PRX* (MIM 605725) encoding periaxin (Einarsdottir et al. 2016); *FNDC1* (MIM 609991) at 6q25.3 (van Ingen et al. 2016); and rs76488276 at 16p12.3 which is ~94kb away from innate immune gene *GP2* (MIM 602977; Li et al. 2017). In the largest GWAS to date including >120,000 European-descent individuals (Pickrell et al. 2016; Tian et al. 2017), 15 risk variants were identified, including four SNPs that were coding and/or intronic but in linkage disequilibrium with coding variants. However the heritability estimated to be due to these common variants is low at ~1% (Tian et al. 2017).

On the other hand, more studies have been done for the otitis media transcriptome, although these were mostly done using microarrays in rodent models and cultured human middle ear epithelial cells (HMEEC). In these studies an acute otitis media-like condition was induced with *Streptococcus pneumoniae* (Spn), non-typeable *Haemophilus influenzae* (ntHI), influenza A virus, TLR gene knockdown, particulate matter, or lipopolysaccharide (Li et al. 2003; Li-Korotky et al. 2004; Leichtle et al. 2009a, 2009b, 2012; Lee et al. 2011; Preciado et al. 2013; MacArthur et al. 2013; Kurabi et al. 2015; Hernandez et al. 2015). In ntHI-inoculated mice, top upregulated genes included inflammatory cytokines *Cxcl1*, *Cxcl2* and *IL-6* (Preciado et al. 2013; MacArthur et al. 2013; Hernandez et al. 2015). Differential expression of these genes were likewise detected in *Tlr*$^{-/-}$ mice, treatment with particulate matter, influenza infection and aging (Leichtle et al. 2012; Nielsen et al. 2016; Kim et al. 2016; Tong et al. 2004; Song et al.

2013). Gene ontology and network analyses identified genes involved in NFKB signaling, innate and immunoglobulin-mediated immune response, inflammatory response, complement activation and cytokine activity (MacArthur et al. 2013; Hernandez et al. 2015; Song et al. 2011). However the expression of these pro-inflammatory cytokines and enrichment of these pathways are not unique to middle ear but are also seen in various inflammatory processes in the nose, lung, and colon and in autoimmune diseases such as diabetes and rheumatoid arthritis (Bartling et al. 2009; Sadighi Akha et al. 2013; Ong et al. 2016; Chen et al. 2016; Vozarova et al. 2003; Kishimoto 1992). Nonetheless these studies increased our knowledge of multiple otitis media-related genes and pathways in a time- and context-dependent manner.

Pichichero et al. conducted two transcriptome studies using serum samples from children with culture-verified acute otitis media pre- and post-infection (Liu et al. 2012, 2013; Pichichero et al. 2016). Genes for host immune response such as complement activation, TLR, and cytokines were differentially expressed in Spn- and ntHI-infected children (Liu et al. 2012, 2013). Differential expression of genes for antimicrobial activity according to pathogen were suggested to correlate with less local inflammation and systemic illness during acute otitis media due to ntHI vs. Spn (Pichichero et al. 2016). Genes encoding lactotransferrin and peptidoglycan recognition protein were downregulated in Spn-infected children (Liu et al. 2012); both proteins are abundantly secreted in the apical air-liquid interface of mouse middle ear epithelium (Mulay et al. 2016). In ntHI-infected children, *STAT1* (MIM 600555) and *PTGS2* (MIM 600262) were downregulated (Liu et al. 2013), which was inconsistent with their upregulation in ntHI-treated mice and influenza-infected HMEECs (MacArthur et al. 2013; Tong et al. 2004),

possibly in part due to the small sample size (*n*=4) per study (Liu et al. 2012, 2013; Pichichero et al. 2016)

Our previous discovery of *A2ML1* (MIM 610627), which encodes alpha-2-macroglobulin-like-1, as an autosomal dominant gene for otitis media susceptibility suggested that rare variants play a role in otitis media pathology (Santos-Cortez et al. 2015). An indigenous Filipino population with a ~50% prevalence of otitis media was found to have an *A2ML1* duplication variant as the strongest predictor for disease (Santos-Cortez et al. 2016b). The same duplication variant was also identified to be associated with otitis-prone status in US children (Santos-Cortez et al. 2015). The duplication variant is predicted to cause aberrant coding of alpha-2-macroglobulin-like-1, a middle-ear-localized protein that may play a role in mitigating mucosal damage during infection and bears close structural resemblance to alpha-2-macroglobulin (A2M), which is a known inflammatory marker in the middle ear and oral cavity (Santos-Cortez et al. 2015). Salivary A2M is increased during inflammatory conditions in the oral cavity, such as gingivitis and periodontal disease (Pederson et al. 1995). Furthermore in a microbiome study, indigenous Filipino carriers with the *A2ML1* duplication and otitis media harbor bacterial pathogens that are commonly associated with dental and oropharyngeal infections e.g. Fusobacteria and Bacteroidetes (Santos-Cortez et al. 2016a), suggesting the possibility of A2ML1-related pathophysiologic processes in the oral cavity.

Here we report novel *A2ML1* variants from exome and Sanger sequence data of Filipino, Finnish, Pakistani and US patients with otitis media. We further describe *A2ML1* variants in relation to gene transcription and oral cavity conditions in indigenous Filipinos. Lastly using RNA-sequence analyses we demonstrate that upregulation of

A2ML1 is correlated with differential expression of multiple genes, particularly genes within keratinocyte and epidermal cell differentiation pathways.

Methods

Study participants

Ethical approval of this study was obtained from: the University of the Philippines Manila Research Ethics Board; the National Commission on Indigenous Peoples, the Institutional Review Board (IRB) of the Helsinki University Hospital; IRB of the University of Maryland School of Medicine; IRB of the University of Texas Medical Branch (UTMB) Galveston; IRB of the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan; and the Colorado Multiple IRB. For the indigenous Filipino population, community consent was obtained prior to study initiation. Individual informed consent was given by all adult participants and parents or guardians of children enrolled in the study.

The indigenous Filipino (Negrito) population is a relatively closed community resulting in extensive intermarriage within six founding families that can be traced genealogically by oral history to 6-7 generations ago. Few individuals who are from other Negrito tribes from adjacent islands married into the community. Due to their physical features of short stature, darkly pigmented skin, curly hair and flat noses, the community has suffered racial segregation from the general Filipino population, resulting in limited opportunities for education, economic advancement, socio-cultural assimilation and health care access. Their community is protected by the government, allowing access

only to researchers who have fulfilled both the community's and the government's requirements for conducting research projects.

For the indigenous community, otitis media was diagnosed based on otoscopic findings at last examination. Chronic otitis media was diagnosed for eardrum perforations with smooth edges, usually with mucoid or mucopurulent discharge and thickened middle ear mucosa. Acute otitis media was diagnosed for hyperemic eardrums with or without perforation or discharge, while otitis media with effusion was identified if with dull non-hyperemic intact eardrums with poor mobility or visible fluid behind the eardrum. Healed otitis media was noted for previously diagnosed chronic, acute or effusive otitis media that has resolved on follow-up examination, or if with healed perforations or eardrum scarring. An individual with chronic, acute, effusive or healed otitis media was labeled as affected with otitis media. Of 135 individuals with DNA samples who were examined by otologists for otitis media, fifty agreed to be checked by dentists for gum disease and dental caries. For the dental exams, gingivitis is defined as gum inflammation with clinical signs and symptoms of bleeding and swelling, with probing depths at 1-3 mm. Extensive review of systems during medical history and physical examination of different parts of the body including skin ruled out additional features that may be part of a syndrome, immunodeficiency or other genetic disease.

From an indigenous community of ~200 individuals, 135 (67.5%) provided saliva samples for DNA isolation using Oragene DNA Collection Kits (DNAgenotek, Ottawa, Ontario, Canada). Of these 135 individuals with DNA samples, 124 (91.9%) have known relations that can be traced to a single pedigree. DNA samples were isolated from saliva using the manufacturer's protocol. An additional 29 Filipino cochlear implantees

provided DNA samples isolated from blood for a study on genetic variants for hearing impairment (Chiong et al. 2013, 2018; Truong et al. 2019).

The Finnish families (Hafrén et al. 2012; Einarsdottir et al. 2016) were ascertained from the Helsinki University Hospital upon referral of the proband for otitis media. Finnish patients were considered positive for otitis media if they had insertion of tympanostomy tubes, effusive otitis media for >2 months, or recurrent otitis media (i.e. >3 episodes in 6 months or >4 episodes in 12 months).

For Pakistani families with otitis media, detailed interviews were conducted with family members to gather information on pedigree structure, comorbidities, onset of disease and initial symptoms. The clinical diagnosis was based on ear discharge and air/bone conduction audiometry. The different groups of study participants are further described in Supp. Table S1.

Exome and Sanger sequencing

Six DNA samples from indigenous Filipinos with otitis media were submitted for exome sequencing at the University of Washington Center for Mendelian Genomics (UWCMG) on an Illumina HiSeq. Sequence capture was performed in solution with either the Roche NimbleGen SeqCap EZ Human Exome v.2.0 or the Big Exome 2011 Library. Fastq files were aligned to the hg19 human reference sequence using Burrows-Wheeler Aligner (BWA; Li & Durbin, 2009, 2010) to generate demultiplexed BAM files. Realignment of indel regions, recalibration of base qualities, and variant detection and calling were performed using the Genome Analysis Toolkit (GATK; McKenna et al. 2010) to produce VCF files. Annotation was performed with SeattleSeq. Two *A2ML1* (RefSeq

NM_144670.5) variants, a duplication and a splice variant, identified from exome data were Sanger-sequenced using the 135 DNA samples from indigenous Filipinos. Available exome sequence data from 29 cochlear implantees from the general Filipino population were also examined for *A2ML1* variants (Chiong et al. 2018). Clinical data of *A2ML1* variant carriers were then checked for otitis media diagnoses. For the Filipino population, identified *A2ML1* variants were Sanger- sequenced using >180 DNA samples from unrelated individuals from the Cebu Longitudinal Health and Nutrition Survey cohort, which were not ascertained for otitis media (Adair et al. 2011).

DNA isolated from blood samples of 234 individuals with otitis media from 218 Finnish families were also submitted for exome sequencing at the University of Washington Northwest Genomics Center, and using the Roche NimbleGen SeqCap EZ Human Exome v.2.0 library, processed as described above. Identified *A2ML1* variants were Sanger-sequenced in the probands and the rest of family members (Supp. Fig. S1).

From all participating family members of 16 Pakistani families with otitis media, peripheral blood samples were collected for DNA extraction. All coding exons of *A2ML1* were Sanger-sequenced in two families. For 14 additional families, a DNA sample of an affected individual was submitted for exome sequencing. Genomic libraries were recovered for exome enrichment using the Agilent SureSelect Human Expanded All Exon V5 (62 Mb) kit. Libraries were sequenced on an Illumina HiSeq. 4000 with average 100× coverage. Alignment and variant calling were likewise performed using BWA and GATK, respectively. Sanger sequencing of the *A2ML1* c.3676_3677delGC variant was performed for the rest of family members with DNA samples from two families PKOM-10 and PKOM-15 (Supp. Fig. S2).

Previously we Sanger-sequenced all 35 coding exons of *A2ML1* using DNA samples from 123 otitis-prone children who were ascertained at UTMB (Patel et al. 2006; Santos-Cortez et al. 2015). These children were considered otitis-prone based on the following criteria: first episode of acute otitis media at <6 months; >3 episodes of acute otitis media within a 6-month period; >4 episodes of acute otitis media within a 12-month period; >6 episodes by 6 years old; or tympanostomy tube surgery for recurrent or persistent otitis media (Patel et al. 2006). In our previous publication, *A2ML1* variants in these children were selected only if (1) it is the most deleterious variant even though there are multiple variants observed in the same child, (2) is absent in controls particularly if missense, and (3) if with scaled Combined Annotation Dependent Depletion (CADD) score >15 plus damaging prediction by at least two bioinformatics tools. For this report the Sanger sequence data from these otitis-prone children were reviewed for additional *A2ML1* variants based on less stringent criteria. The decision to use less stringent criteria is based on our recent observations of common variants that are deemed polymorphisms due to higher MAF but are shown to be involved in otitis media susceptibility, and of otitis media patients carrying multiple variants from the same gene or multiple genes despite observation of autosomal dominant inheritance with reduced penetrance in families (Santos-Cortez et al. 2018).

For all identified variants whether previously published or novel, variants were classified as pathogenic/likely pathogenic or variant of unknown significance (VUS) based on current criteria from the American College of Medical Genetics (ACMG; Richards et al. 2015) using the Genetic Variant Interpretation Tool.

Bioinformatics, linkage and mixed model analyses

From exome or Sanger sequence data, variants were considered further if they have MAF less than 0.02 in the general population, have a scaled CADD score greater than 3, and are considered damaging by at least one additional bioinformatics tool (Table 1). For the Finnish, Pakistani and US populations, MAF was derived from the genome Aggregation Database (gnomAD) using Finnish, South Asian, non-Finnish European or Latino allele data, when appropriate.

For the two *A2ML1* variants c.3676_3677delGC and c.4061+1G>C, two-point linkage analysis was performed using Superlink (Fishelson & Geiger, 2002). For the frameshift variant, linkage analysis was performed using variant MAF of 0.07, disease allele frequency of 0.01, and two modes of inheritance, namely: (a) affecteds-only with autosomal dominant inheritance, 90% penetrance and 5% phenocopy rate; and (b) autosomal recessive inheritance with full penetrance and no phenocopies (Supp. Fig. S2). For the *A2ML1* c.4061+1G>C variant in the indigenous population, two-point linkage analysis was performed using an affecteds-only model with autosomal dominant inheritance, 90% penetrance, 5% phenocopy rate, disease allele frequency of 0.01 and variant MAF of 0.000001 (Supp. Fig. S3).

Fisher exact test was used to test associations between *A2ML1* variants, otitis media and/or dental findings in the indigenous Filipino population. For mixed model analysis testing the association between otitis media and multiple variables including age, sex, carriage of at least one *A2ML1* variant and gingivitis as fixed effects, grouping by family branch or household was used as a random effect variable.

RNA sequencing and analysis

For RNA studies, saliva samples were collected from nine indigenous Filipinos using the Oragene·RNA RE-100 kit. An additional 23 saliva samples were collected from otitis media patients undergoing surgery at the Children's Hospital Colorado and the University of Colorado Hospital (Supp. Tables S1-S2) using Oragene·RNA kits. Salivary RNA was extracted according to the manufacturer's protocol.

RNA samples were analyzed on an Agilent 2200 Tapestation and processed with the NuGen Trio RNA-Seq Kit at the University of Colorado Denver Genomics and Microarray Core. RIN values ranged from 4.5-7.7 (+ S.D. 0.92). Sequencing libraries were sequenced on an Illumina HiSeq. 4000 generating 50 bp single-end reads, with Filipino samples pooled at equimolar concentrations and sequenced in a single lane while Colorado samples were pooled and sequenced across three lanes. Reads were trimmed and adaptor sequences were removed using the FASTX-Toolkit (v0.0.13) prior to alignment to the hg38 human genome (GENCODE release 24) using STAR v2.5.3a (Dobin et al. 2013). Aligned reads were summarized at the gene level using featureCounts v1.5.2 with default parameters (Liao et al. 2014). For Filipino RNA samples, raw counts were analyzed according to variant carriage using Wilcoxon tests in R.

For the Colorado samples, count values from the technical replicates per sample were summed. Genes with an average count value of <3 were discarded resulting in ~12,000 remaining genes. The filtered count matrix was input to DESeq. 2 v1.20.0 (Love et al. 2014) in R v3.5.1 for differential expression analysis, while comparing high- vs.

low-*A2ML1*-expressors. For correlation analysis, the normalized count matrix from DESeq. 2 was rlog-transformed, then correlation was performed for *A2ML1* vs. all other genes using the Spearman method in R. Heatmap visualization of DESeq. 2 results was performed using the 'pheatmap' package in R. Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified using the Generally Applicable Gene-set Enrichment (GAGE v2.30.0) package in R (Luo et al. 2009). Log-2 fold changes calculated by DESeq. 2 were input to GAGE and processed using default parameters.

1.1 For validation of differentially expressed genes, cDNA was generated from 23 RNA samples from Colorado using the Invitrogen SuperScript IV protocol. Each cDNA sample was used for qPCR on a Bio-Rad machine (Hercules, CA, USA) in triplicate using the Applied Biosystems PowerUp SYBR green master mix and each set of primers for *A2ML1* (NM_144670.5), differentially expressed genes *AHNAK* (NM_001620.2) and *RND3* (NM_005168.4), and *ACTB* (NM_001101.3) as control. Fold change was determined according to ΔCT values.

Results

Sixteen novel, rare or low-frequency *A2ML1* variants were identified from exome data of individuals with otitis media (Table 1). Seven of these variants were observed in UTMB otitis-prone probands. Notably two probands UTMB-959 and UTMB-1031 have 2-3 *A2ML1* variants each with at least one as a novel variant (Table 1). Most of these variants found in UTMB probands did not pass previously set criteria due to lower CADD scores using the earlier software version and/or having only one damaging prediction, for example, c.2228C>T (p.(Pro743Leu)) in UTMB-1027 and c.2971G>C (p.(Ala991Pro)) in UTMB-1018. However due to lack of additional evidence, these novel variants along

with some previously identified variants in UTMB probands were classified as VUS (Table 1; Fig. 1).

Four novel variants were identified as heterozygous in Finnish otitis media patients with exome data, including two missense and two non-canonical splice variants, all of which are predicted to be damaging (Table 1; Supp. Fig. S1). Two variants c.971-8C>T and c.1308A>C (p.(Gln436His)) occurred uniquely in a single Finnish proband. The c.2197T>C (p.(Phe733Leu)) was found in two individuals and has a MAF=0.004 in the Finnish population, while the low-frequency splice variant c.2713-8C>A was observed in seven Finnish families (Table 1). The Finnish probands carrying *A2ML1* variants have no known syndromic features.

From the sequence data of 16 Pakistani families with otitis media, affected individuals from two families carried a frameshift variant c.3676_3677delGC (p.(Ala1226Glnfs*34)) that is predicted by MutationTaster (Schwarz et al. 2010) to be a polymorphism though leading to nonsense-mediated decay (Supp. Fig. S2). This variant has MAF=0.07 in gnomAD South Asian alleles and does not fully co-segregate with otitis media in the two Pakistani families using an autosomal dominant model or affecteds-only analysis (Supp. Fig. S2). Because three affected individuals of family PKOM-15 are homozygous for the frameshift variant and has otitis media from early childhood, the variant potentially co-segregates with autosomal recessive otitis media in branch 2 of family PKOM-15. However the LOD score is deflated compared to the maximum LOD score that is expected given pedigree branch structure and autosomal recessive inheritance with full penetrance (Supp. Fig. S2). All other *A2ML1* variants identified in the sequence data of the Pakistani families were frequent (MAF>0.20) or

deemed non-damaging to function. For these families, autosomal recessive variants that remain to be identified likely play a greater role in otitis media pathology.

Four *A2ML1* variants were identified in three Filipino children who had cochlear implantation for congenital hearing impairment and who also have a history of otitis media. One child was heterozygous for two *A2ML1* variants, namely c.10C>T (p.(Gln4*)) and c.2329G>A (p.(Gly777Arg)). Of these two variants, the stop variant is classified as pathogenic while the missense variant is a VUS (Table 1; Fig. 1). Unfortunately we have no parental DNA for testing if these two variants are compound heterozygous or in linkage disequilibrium. At age 8 months, a year prior to cochlear implantation, the child with these two variants had bilateral type C tympanograms. This patient is also homozygous for *SLC26A4* (MIM 605646) c.706C>G (p.Leu236Val) which is the known cause for his profound hearing loss and enlarged vestibular aqueducts (Chiong et al. 2018). The second Filipino cochlear implantee is heterozygous for *A2ML1* c.2012T>C (p.(Leu671Pro)) and had a flat tympanogram for the right implanted ear 14 months post-surgery at age 6 ½ years. She is also homozygous for a splice variant c.2301+1G>T within *OTOA* (MIM 607038), likely the genetic cause of her congenital profound hearing loss (Truong et al. 2019). The third cochlear implantee is heterozygous for both a variant in hearing loss gene *COL4A3* (MIM 120070) c.764C>T (p.(Thr255Met)) and *A2ML1* c.4061+1G>C. All four *A2ML1* variants identified in Filipino cochlear implantees were predicted to be damaging and were absent in the general Filipino population (Table 1).

Previously the *A2ML1* c.2478_2485dupGGCTAAAT (p.Ser829Trpfs*9) variant was identified in the exome sequence data of two second-cousins from the indigenous

Filipino population (Santos-Cortez et al. 2015). Out of 135 individuals (Table 2), this duplication variant is heterozygous in 62 and homozygous in eight indigenous Filipinos, and of these 70 variant carriers, 49 (70%) have otitis media. However, 33 individuals who were wildtype for the duplication variant also had otitis media. We therefore submitted for exome sequencing DNA samples from four additional individuals who were wildtype for the duplication variant and come from different subpedigrees within the community. In two individuals with exome data, the *A2ML1* splice variant c.4061+1G>C was identified. Of 135 indigenous individuals (Table 2), one is homozygous and 24 are heterozygous for the splice variant, including nine that are compound heterozygous for the two *A2ML1* variants. Majority of those who carry the splice variant can be connected by three subpedigrees based on known relations (Supp. Fig. S3). Two-point linkage analysis for the splice variant resulted in a LOD score of 3.2 (θ=0; Supp. Fig. S3). In total, 86 (63.7%) of the screened population carry *A2ML1* variants, and of these 60 (69.7%) have otitis media (Table 1). Conversely of 82 individuals with otitis media, 60 (73.2%) carry *A2ML1* variants. Therefore among 135 indigenous Filipinos, the odds ratio for an *A2ML1* variant carrier having otitis media is 2.8 (95%CI: 1.3, 6.2; p=0.006; Table 2).

Among 50 indigenous Filipinos with dental examinations, 28 (56%) had otitis media and 34 (68%) carried *A2ML1* variants, which is comparable to the bigger cohort (Table 2). In addition, 52% were male with a mean age of 13.64 years (range 4 months - 47 years). Of these 50 individuals, 44 had dental caries, 24 had gum bleeding and 38 were diagnosed with gingivitis. None of these dental conditions were associated with *A2ML1* variants, however gingivitis was associated with otitis media (OR=5.6, 95%CI:

1.1, 37.5; p=0.02). In mixed models analysis, in which family branch or household was analyzed as a random effect, otitis media was associated with both gingivitis (p=0.0007) and *A2ML1* variants (p=0.048). Neither age nor sex was associated with otitis media, consistent with previous findings (Santos-Cortez et al. 2016b).

To further determine the effect of the *A2ML1* duplication and splice variants in the indigenous Filipinos, RNA-sequencing was performed using salivary DNA from nine indigenous individuals, two of whom are wildtype, five are heterozygous, one compound heterozygous and one homozygous. RNA counts showed a decrease in salivary *A2ML1* expression according to carriage of *A2ML1* variants (Fig. 2A).

Because *A2ML1* variants related to otitis media are rare, in order to study the effects of changes in *A2ML1* expression in otitis media, salivary DNA samples from 23 otitis media patients from Colorado were also submitted for RNA-sequencing. For this analysis, based on the inflection of the count plot for *A2ML1* in the Colorado samples (Supp. Fig. S4), differential expression analysis was initially performed using a threshold count of 33 for the Colorado samples, dividing the group into low- and high-*A2ML1*-expressors. Out of 12,107 post-filtered genes in salivary RNA, differential expression analysis using DESeq. 2 identified 745 (6.2%) genes at FDR-adjusted p<0.05, with 442 upregulated and 303 downregulated genes in high-*A2ML1*-expressors (Supp. Tables S3-S4). Because *A2ML1* count values ranged from ~5-550 (+S.D.125), we also performed a genome-wide correlation analysis and *A2ML1* values were compared against all other genes. A total of 41 genes were highly correlated with *A2ML1* (r >+0.81, Bonferroni-corrected p<0.05; Table 3). Of note, 14 genes overlap among the top differentially expressed genes identified by both DESeq. 2 and correlation analysis, including: *MUC21*

(MIM 616991); *PPL* (MIM 602871); *SPINK5* (MIM 605010); *LPIN1* (MIM 605518); *KRT13* (MIM 148065); *SCEL* (MIM 604112); *CRNN* (MIM 611312); *TGM3* (MIM 600238); *LMO7* (MIM 604362); *MAL* (MIM 188860); *MYO5B* (MIM 606540); *SASH1* (MIM 607955); *FLG* (MIM 135940); and *TMPRSS11B* (Fig. 3; Table 3). Of the 41 correlated genes, *AHNAK* and *RND3* were also found to be upregulated in indigenous Filipino *A2ML1*-variant carriers compared to wildtype (Fig. 2B-2C). Validation using qPCR on the Colorado RNA samples confirmed that *RND3* is significantly enriched in high-*A2ML1*-expressors (Table 4).

When genes with log-2-fold differential expression >2 were analyzed using the GAGE package, 88 KEGG pathways and gene ontology terms were enriched ($p<0.05$; Supp. Table S5). In particular, keratinocyte differentiation ($p=2.9\text{x}10^{-6}$) and epidermal cell differentiation ($p=3.2\text{x}10^{-5}$) were enriched in high-*A2ML1*-expressors (Supp. Table S5).

Because the mean age of low-*A2ML1*-expressors was noticeably higher than high-*A2ML1*-expressors (Supp. Table S2), we reanalyzed the Colorado dataset excluding samples with age >10 years (low-*A2ML1*-expressors mean 3.27 years vs. high-*A2ML1* expressors mean 1.71 years). Differential expression analysis yielded similar results, albeit fewer significant genes were detected i.e. 371 upregulated and 157 downregulated genes in *A2ML1*-high-expressors (Supp. Table S6). Many of the same KEGG and GO terms were upregulated in *A2ML1*-high-expressors, however downregulated KEGG and GO terms were different (Supp. Table S6). We attribute these differences to the reduced number of significantly dysregulated genes. Regardless we conclude that the age range in

the low-*A2ML1*-expressors does not account for any of the dysregulated genes we observed.

Discussion

In this study we identified sixteen novel *A2ML1* variants in otitis media patients, two of which are pathogenic based on ACMG criteria (Table 1; Fig. 1), further providing evidence to support a role for *A2ML1* in middle ear mucosal pathology. Interestingly the variants that are deemed pathogenic are loss-of-function variants that are mostly predicted by MutationTaster to result in nonsense-mediated decay (Table 1). This is further supported by decreased transcription levels of *A2ML1* due to the duplication and c.4061+1G>C variants (Fig. 2A). All the loss-of-function variants are also predicted to remove important domains, with at least the receptor-binding domain (RBD) being affected (Fig. 1). Based on the known crystallographic structure of homotetrameric A2M complex, after induction the RBD of one monomer protrudes into the circulation which results in the recognition of the tetramer by cell-surface receptors and leads to endocytosis, lysosomal degradation and clearance of the protease inhibitor complex along with the proteases it trapped (Marrero et al. 2012). Loss of the RBD is therefore predicted to result in lack of recognition of A2ML1 and failure of clearance of proteases for removal in order to prevent undue damage to mucosa. In our previous study, we showed that some missense variants are predicted to result in more subtle torsional changes in the macroglobulin (MG) or thiol-ester domains that interact to form the tetrameric structure (Santos-Cortez et al. 2015). In particular, most of the *A2ML1* variants identified in otitis media patients occur within the MG6-MG7 domains, and these MG6-MG7 domains are required to close the superhelical structure in order to trap proteases that are baited by the

bait-region domain (BRD) within MG6 (Marrero et al. 2012). Because most of the missense variants identified were seen in single probands and were therefore classified as VUS, identification of additional otitis media patients with these specific variants will aid in establishing variant pathogenicity.

Three of the novel *A2ML1* variants were recurrent: the splice variant c.4061+1G>C variant was identified in the indigenous Filipino population and in a cochlear implantee with otitis media from the general Filipino population; and a missense variant c.2197C>T (p.(Phe733Leu)) and a splice variant c.2713-8C>A in Finnish patients with otitis media. Here we also identified a frameshift variant in two Pakistani families, however this variant did not co-segregate with otitis media suggesting both intra-familial genetic heterogeneity (Rehman et al. 2015) and unidentified otitis media susceptibility variants with likely autosomal recessive inheritance (Supp. Fig. S2). In our previous study the *A2ML1* duplication variant c.2478_2485dupGGCTAAAT that was initially identified to co-segregate with otitis media in the intermarried indigenous Filipino population was also genome-wide significant in three European-American and Hispanic-American children (Santos-Cortez et al. 2015; Table 1). Taken together, these findings suggest that *A2ML1* variants conferring otitis media susceptibility are population-specific.

Although *A2ML1* variants in general confer susceptibility to autosomal dominant nonsyndromic otitis media, the consanguineous Pakistani family PKOM-15 has additional cognitive, cranial and cardiac anomalies that do not co-segregate with the c.3676_3677delGC (p.(Ala1226Glnfs*34)) variant or otitis media. These additional phenotypes are not exactly the same but have some overlaps with the clinical features of

intellectual disability, craniofacial and cardiac defects i.e. pulmonary valve stenosis in Noonan-like syndrome due to rare *A2ML1* variants p.Arg592Leu, p.Arg802Leu and p.Arg802His (Vissers et al. 2015). Note that *A2ml1*-mutant zebrafish also had broad heads and failed cardiac looping (Vissers et al. 2015). *A2ML1* variants have also been associated with hypertension, however the variants identified for hypertension do not overlap with otitis media-related variants, except for two variants p.Val296Ala and p.Arg893* which were observed separately in US probands with otitis media and in Europeans with hypertension (Table 1; Fig. 1; Santos-Cortez et al. 2015; Surendran et al. 2016). This may suggest that allelic heterogeneity for *A2ML1* contributes to phenotypic heterogeneity; for example, *A2ML1* variants identified for otitis media cluster within the MG6 and MG7 domains while variants for hypertension tend to lie within the MG3 domain and the TED region of the CUB domain, alluding to differences in genotype-phenotype effects (Fig. 1; Surendran et al. 2016). On the other hand, it might also mean that *A2ML1* variants confer susceptibility to otitis media in childhood and hypertension in later life.

While potential pleiotropic effects of *A2ML1* remain to be fully resolved, our data showed that *A2ML1* variants are not associated with oral disease. Previously A2M protein was found to be higher in gingival crevicular fluid of patients with gingivitis and chronic periodontitis, but whether A2M plays a role in pathogenesis or is merely a marker for inflammation is unknown (Ertugrul et al. 2013). We first hypothesized that the structural similarity between A2M and A2ML1 might translate to an increased risk for dental disease conferred by *A2ML1* variants. However in this study *A2ML1* variants were not associated with dental conditions including gum disease. Saliva possesses a vast array

of enzymes, immunoglobulins, glycoproteins and cystatins that preserve the equilibrium of the microbiological flora in the oral cavity and are crucial in maintaining oral health (Amerongen & Veerman, 2002). This could account for why a defect in a single immunoregulatory protein in saliva would not necessarily lead to breakdown of protective barriers and consequently manifest as dental disease. It is also possible that in the head and neck region the mucosal protection conferred by A2ML1 is specific to the middle ear despite the upper airway being contiguous with the oral cavity (Santos-Cortez et al. 2015). Such compartmentalization of tissue-site expression of immune factors has been documented in other parts of the body with mucosal surfaces (Burgener et al. 2013).

On the other hand, both gingivitis and *A2ML1* variants contribute independently to otitis media status. These significant effects are observed independent of age, and with correction for household or close familial relations as a random effect. When gum inflammation is present, retrograde movement of pathogens from the oral cavity into the nasopharynx and eventually the middle ear may contribute to otitis media pathogenesis. For example, clonal similarity between *Fusobacterium nucleatum* isolated from middle ear effusion and the oropharynx was documented previously (Topcuoglu et al. 2012). In middle ears of *A2ML1* variant carriers, we observed a nominally significant increase in relative abundance of Fusobacteria and Bacteroidetes, which are more commonly identified as oral cavity or oropharyngeal pathogens (Santos-Cortez et al. 2016a; Suzuki et al. 2015; Yang et al. 2014). The additional finding of gingivitis as an independent risk factor for otitis media further supports the oral cavity as a potential source of otitis media pathogens in middle ears with weaker mucosal protection due to A2ML1 defects (Santos-Cortez et al. 2015, 2016a). It also suggests that prevention of gum disease may be an

effective public health measure towards decreasing otitis media burden in the indigenous Filipino population.

Another significant finding is the co-upregulation of genes, including genes involved in keratinocyte and epidermal cell differentiation, when *A2ML1* is upregulated. One of the byproducts of chronic otitis media is cholesteatoma, which is a collection of squamous debris encapsulated by keratinized epithelium in the middle ear (Maniu et al. 2014). In contrast to pseudostratified ciliated or simple squamous epithelium of healthy middle ear mucosa, the cholesteatoma capsule consists of keratinized stratified squamous epithelial matrix and a collagenous submatrix with inflammatory cells (Lim & Saunders, 1972). Given that many of the cellular, biochemical and regulatory factors favoring cholesteatoma growth, expansion and bony erosion remain unknown, the differentially expressed genes due to lower or higher *A2ML1* expression may provide clues to the process of cholesteatoma formation.

Interestingly *RND3* and *AHNAK* were shown to be upregulated in otitis media patients, whether in high-*A2ML1*-expressors or in *A2ML1* variant carriers with lower *A2ML1* expression. This may suggest that *RND3* and *AHNAK* are involved in middle ear homeostasis in response to changes in *A2ML1* expression. *RND3* encodes Rho GTPase 3/RhoE which disorganizes the actin cytoskeleton by inhibiting ROCK-1, RhoA and Rac signaling while increasing cytokines NFKB and IRAK (Guasch et al. 2007). Interestingly Rac activates JNK, and in the infected mouse middle ear JNK inhibition resulted in decreased mucosal hyperplasia (Furukawa et al. 2007). In addition, injured astrocytes treated with Fasudil, a ROCK inhibitor, had widespread AHNAK labeling and downregulated protein degradation pathways, indicating a healthy state (O'Shea et al.

2015). Likewise treatment of the inflamed gut with Rnd3 reduced microvascular permeability (Breslin et al. 2016). ROCK inhibition is actually a common method for forming a confluent layer of HMEECs in culture studies (Mulay et al. 2016), however this application has not been considered for augmenting therapies for otitis media.

Overall our studies further support a role for *A2ML1* in otitis media and reveal novel variants, genes and pathways related to otitis media pathology.

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Web Resources

Burrows-Wheeler Aligner, bio-bwa.sourceforge.net

ClinVar, www.ncbi.nlm.nih.gov/clinvar (SCV000882552 - SCV000882560)

Combined Annotation Dependent Depletion, cadd.gs.washington.edu

DESeq. 2 v1.20.0, bioconductor.org/packages/release/bioc/html/DESeq. 2.html

FastX-ToolKit v0.0.13, hannonlab.cshl.edu/fastx_toolkit

featureCounts v1.5.2, bioinf.wehi.edu.au/featureCounts

GAGE v2.30.0, bioconductor.org/packages/release/bioc/html/gage.html

GENCODE, www.gencodegenes.org

Gene Ontology Consortium, www.geneontology.org

Genetic Variant Interpretation Tool,

www.medschool.umaryland.edu/Genetic_Variant_Interpretation_Tool1.html/

Genome Aggregation Database, gnomad.broadinstitute.org

Genome Analysis Toolkit, software.broadinstitute.org/gatk/

GWAS Catalog, www.ebi.ac.uk/gwas/

KEGG, www.genome.jp/kegg/

MutationAssessor, mutationassessor.org/r3/

MutationTaster, www.mutationtaster.org

Online Mendelian Inheritance in Man, www.omim.org

Pheatmap v1.0.10, CRAN.R-project.org/package=pheatmap

PolyPhen-2, genetics.bwh.harvard.edu/pph2/

PROVEAN, provean.jcvi.org

R v3.5.1, www.r-project.org

SeattleSeq Annotation, snp.gs.washington.edu/SeattleSeqAnnotation151

STAR v2.5.3a, github.com/alexdobin/STAR

Superlink Online SNP 1.1, cbl-hap.cs.technion.ac.il/superlink-snp/

UCSC Genome Browser, genome.ucsc.edu

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Table 1. *A2ML1* Variants Identified in Multi-ethnic Families and Probands with Otitis Media

Cohort - Patient ID	hg19 chr12 Coordinate	Variant (NM_144670.5)†	Control MAF‡	Protein Domain	CADD	Mutation Taster	PolyPhen-2 HumDiv	PROVEAN	SIFT	MutationAssessor
I. Pathogenic/likely pathogenic										
CIFIL-11	8975257	**c.10C>T (p.(Gln4*))**	0	MG1	35.0	D(NMD)	--	--	--	--
UTMB-1039	8990070	c.763C>T (p.(Gln255*))	0	MG3	35.0	D(NMD)	--	--	--	--
IPOM, UTMB-959/969/970	9004827	c.2478_2485dupGGCTAAAT (p.(Ser829Trpfs*))	0	MG7	--	D(NMD)	--	--	--	--
UTMB-1031	9009825	c.2914G>T (p.(Glu972*))	0	CUB/TED	44.0	D(NMD)	--	--	--	--
IPOM, CIFIL-21	9020954	**c.4061+1G>C**	0	RBD	25.7	D	--	--	--	--
II. Variants of unknown significance										
UTMB-1031	8975879	**c.164C>T (p.(Thr55Ile)**	0	MG1	19.6	P	PoD	D	D	M
UTMB-1178, UMN-123	8990963	c.887T>C (p.(Val296Ala))	0.0009	MG3	22.5	P	B	D	D	M
UHF-269	8991701	**c.971-8C>T**	0.0006	MG3	13.9	D	--	--	--	--

UTMB-1017	8991805	c.1067C>G (p.(Pro356Arg))	0	MG4	25.0	P	PoD	D	D	M
UHF-101	8995789	**c.1308A>C (p.(Gln436His))**	0.00005	MG4	15.4	P	B	N	T	M
UTMB-1026	8998818	**c.1683G>C (p.(Gln561His))**	0	MG6	33.0	D	PoD	D	D	M
CIFIL-14	9001494	**c.2012T>C (p.(Leu671Pro))**	0	MG6/BRD	13.3	P	B	D	D/T	L
UTMB-998	9002825	**c.2189G>A (p.(Arg730His)**	0.003	MG6	21.3	P	B	D	T/D	L
UHF-254/255	9002833	**c.2197T>C (p.(Phe733Leu))**	0.004	MG6	23.0	D	B	D	T/D	L
UTMB-1027	9002864	**c.2228C>T (p.(Pro743Leu))**	0	MG6	23.4	P	B	D	T	L
CIFIL-11	9004474	**c.2329G>A (p.(Gly777Arg))**	0	MG7	23.3	D	PrD	D	D	H
UTMB-1031	9004573	c.2428G>A (p.(Ala810Thr))	0	MG7	25.2	D	PrD	D	D	H
UTMB-1019	9004887	**c.2545G>T (p.(Asp849Tyr))**	0	MG7	15.6	P	PoD	D	D	M
UTMB-1030	9006810	c.2677C>T (p.(Arg893*))	0.009	MG7	34.0	D(NMD)	--	--	--	--
7 UHF families	9007368	**c.2713-8C>A§**	0.013	MG7	4.8	D	--	--	--	--
UTMB-1018	9009882	**c.2971G>C (p.(Ala991Pro)**	0	CUB/TED	20.8	P	B	N	D	L
UTMB-	90099	c.3001C>T	0	CUB/	24.5	P	PrD	D	D	M

971	12	(p.(Arg1001Trp))		TED						
UTMB-959	9013882	**c.3491C>T (p.(Ala1164Val))**	0	CUB/TED	27.5	D	PrD	D	D	M
PKOM-10/15	9016563	**c.3676_3677delGC (p.(Ala1226Glnfs*34))**	0.07	CUB/TED	--	P(NMD)	--	--	--	--
UTMB-1027	9027091	c.4292C>T (p.(Ala1431Val))	0	RBD	25.6	D	PrD	D	D	L

Abbreviations: BRD, bait-region domain; CADD, Combined Annotation Dependent Depletion; CIFIL, Filipino cochlear implantee; CUB, complement protein subcomponent C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein 1 domain; IPOM, indigenous Filipino cohort; MAF, minor allele frequency; MG, macroglobulin domain; RBD, receptor-binding domain; TED, thiol ester-containing domain; UHF, Finnish cohort; UMN, Minnesota cohort; UTMB, Texas cohort. MutationTaster: D, disease-causing; NMD, nonsense-mediated decay; P, polymorphism. PolyPhen-2: PrD, probably damaging; PoD, possibly damaging; B, benign. PROVEAN: D, deleterious; N, neutral. SIFT: D, deleterious; T, tolerated ("/" denotes multiple predictions depending on isoform). MutationAssessor: H, high; M, medium; L, low.

†Novel variants are in *bold* font. Known variants were previously reported in Santos-Cortez et al. 2015.

‡For CIFIL and IPOM, control MAF is from the Cebu Longitudinal Health and Nutrition Survey. For UHF, UMN and PKOM, control MAF is from gnomAD Finnish, non-Finnish European and South Asian, respectively. For UTMB, control MAF is either from gnomAD non-Finnish European or Latino populations depending on self-reported ethnicity. UTMB IDs 959, 1030, 1031, 1039 and 1178 are Hispanic while the rest of UTMB IDs are non-Hispanic White. In some cases the gnomAD MAF in another population is higher, e.g. the c.2677C>T (p.(Arg893*)) variant has Latino MAF 0.00009 but has African and non-Finnish European MAF=0.0002.

§Eight individuals with exome data from seven Finnish families carry the c.2713-8C>A variant (Supp. Fig. S1). From the exome data two common SNPs namely rs73037000 (chr12:8987285G>A) and rs1860967 (chr12:9013755C>T) flank the c.2713-8C>A variant, which comprise a short 26,470-bp haplotype found in all eight carriers. However four carriers have various common or low-frequency variants within the haplotype, suggesting that this haplotype is very old and multiple recombinations have occurred within the region.

Table 2. *A2ML1* Genotypes and Otitis Media Status for 135 Indigenous Filipinos

A2ML1 genotype	Normal otoscopy	Otitis media†	Total
Wildtype	*27*	*22*	*49*
Heterozygous, duplication	17	36	53
Heterozygous, splice	5	10	15
Compound heterozygous	1	8	9
Homozygous, duplication	3	5	8
Homozygous, splice	0	1	1
Total with variant‡	*26*	*60*	*86*
Overall total	*53*	*82*	*135*

†Of 60 *A2ML1* variant carriers with otitis media, 22 (36.7%) have chronic otitis media, 23 (38.3%) with healed otitis media, 14 (23.3%) with effusive otitis media, and 1 with acute otitis media based on last exam. In contrast, among 22 wildtype individuals with otitis media, 9 (40.9%) have chronic otitis media, 5 (22.7%) with healed otitis media, another 5 (22.7%) with effusive otitis media and 3 (13.6%) with acute otitis media. Fifteen of the 22 wildtype individuals with otitis media carry the *FUT2* c.604C>T (p.Arg202*) variant which also plays a role in otitis media susceptibility (Santos-Cortez et al. 2018; Supp. Fig. S3).

‡The Fisher exact odds ratio for an *A2ML1* variant carrier having otitis media is 2.8 (95%CI: 1.3, 6.2; p=0.006).

Table 3. Top 41 genes correlated with *A2ML1* in Colorado patients and their known role in mucosal, epithelial or infectious traits

Gene	Spearman R	*p*-value	Known Role in Mucosa/Infection/Epithelium	Reference
EMP1	0.919	3.30E-06	Downregulated in nasal polyps	Yu et al. 2013
SPRR3	0.903	3.28E-06	Upregulated in chronic rhinosinusitis	Ramakrishnan et al. 2017
RND3	0.896	3.19E-06	Forms confluent layer of HMEECs	Mulay et al. 2016
CSTB	0.887	3.02E-06	Upregulated in gingival epithelial cells after contact with *Fusobacterium nucleatum*	Yin & Dale 2007
MUC21	0.886	3.00E-06	Induced by RSV and hMPV	Banos-Lara et al. 2015
RNR2	0.880	2.84E-06	Expressed in olfactory mucosa	Bergstrom et al. 2007
MTRNR2L8	0.876	2.73E-06	--	--
KRT4	0.876	2.73E-06	Upregulated in cholesteatoma	Britze et al. 2014
PPL	0.873	2.60E-06	Autoimmune target in IPF	Taille et al. 2011
SPINK5	0.870	2.50E-06	Otitis media in Netherton syndrome	Renner et al. 2009
TMPRSS11 B	0.869	2.47E-06	Expressed in squamous epithelia of cervix, esophagus and oral cavity	Miller et al. 2014
ECM1	0.867	2.40E-06	Mucosal defect in lipoid proteinosis	Mirancea et al. 2007
ERO1L	0.867	2.40E-06	High expression in gastric cancer with poor prognosis	Seol et al. 2016
LPIN1	0.866	2.36E-06	Downregulated due to high-fat diet along with changes in gastrointestinal mucosa	Zhou et al. 2016
S100A10	0.862	2.23E-06	Interaction with *TRPV6* in airway and gut epithelia	Borthwick et al. 2008
KRT13	0.858	2.09E-06	Mutations lead to white sponge naevus in buccal mucosa	Liu et al. 2011
TMPRSS11 D	0.858	2.09E-06	Protease in airway epithelium	Menou et al. 2017
AHNAK	0.851	1.89E-06	Upregulated in otitis media	This report

SPRR1B	0.848	1.82E-06	Upregulated in chronic rhinosinusitis	Ramakrishnan et al. 2017
SCEL	0.847	1.80E-06	Forms cornified envelope in stratified squamous epithelium	Baden et al. 2005
RNR1	0.845	1.76E-06	--	--
CRNN	0.843	1.73E-06	Downregulated in esophageal cancer	Pawar et al. 2013
CAST	0.840	1.71E-06	Decreases influenza A infection	Blanc et al. 2016
PABPC1	0.837	1.71E-06	Interaction with HSV-1 proteins	Dobrikova et al. 2010
ADAM9	0.836	1.72E-06	Increased in COPD bronchial cells	Wang et al. 2018
ATF6B	-0.836	1.72E-06	--	--
KRT78	0.834	1.74E-06	Expressed in squamous epithelia	Langbein et al. 2016
TGM3	0.833	1.76E-06	Cross-links with HPV proteins	Brown et al. 2006
LMO7	0.832	1.78E-06	Upregulated by *H.pylori* in gastric cells	Lim et al. 2003
AIM1	0.831	1.81E-06	Expressed in EBV-infected cells	Cahir-McFarland et al. 2004
ANXA1	0.831	1.81E-06	Regulates intestinal mucosal injury, inflammation and repair	Babbin et al. 2008
FAM129B	0.831	1.81E-06	Knockout results in delayed healing of wounded skin; keratinocyte-expressed	Oishi et al. 2012
SEMA3F	-0.831	1.81E-06	Regulates neurite growth in lingual epithelium	Vilbig et al. 2004
ANXA2	0.830	1.84E-06	Increased bacterial opsonization and clearance in otitis media	Renner et al. 2016
MAL	0.830	1.84E-06	Methylated in dysplastic cervix	Mersakova et al. 2014
EHF	0.823	2.21E-06	Regulates airway gene expression	Fossum et al. 2017
ERGIC2	0.823	2.21E-06	--	--
MYO5B	0.820	2.47E-06	Mutation causes intestinal microvillus defects	Muller et al. 2008
SASH1	0.816	2.91E-06	Mutation causes lentiginous skin phenotypes	Zhang et al. 2016
FLG	0.813	3.34E-06	Lower expression in cholesteatoma	Nguyen et al. 2014

KLK13	0.810	3.85E-06	In periodontitis *Porphyromonas gingivalis* proteases degrade SPINK6 resulting in loss of KLK13 inhibition	Plaza et al. 2016

HMEEC, human middle ear epithelial cells; RSV, respiratory syncytial virus; hMPV, human metapneumovirus; IPF, idiopathic pulmonary fibrosis; HSV-1, herpes simplex virus-1; COPD, chronic obstructive pulmonary disease; HPV, human papilloma virus; EBV, Epstein-Barr virus.

Table 4. Fold Changes by Gene Based on qPCR ΔCT Values from Colorado patients

Gene	High-Expressors	Low-Expressors	ΔΔCT	Fold Change
A2ML1	5.94	8.45	-2.52	5.72*
AHNAK	5.32	6.08	-0.76	1.69
RND3	4.05	6.53	-2.48	5.58**

*$p<0.05$, **$p<0.01$

Figures

Fig. 1. A2ML1 variants identified in families and probands with otitis media and their occurrence within protein domains. Domain names as in footnote to Table 1. Variants on *top* of the boxed representation of the A2ML1 protein are novel and are included in this report; *below* are previously published (Santos-Cortez et al. 2015). Variants in *bold red* font are pathogenic/likely pathogenic while the rest of variants are of unknown significance.

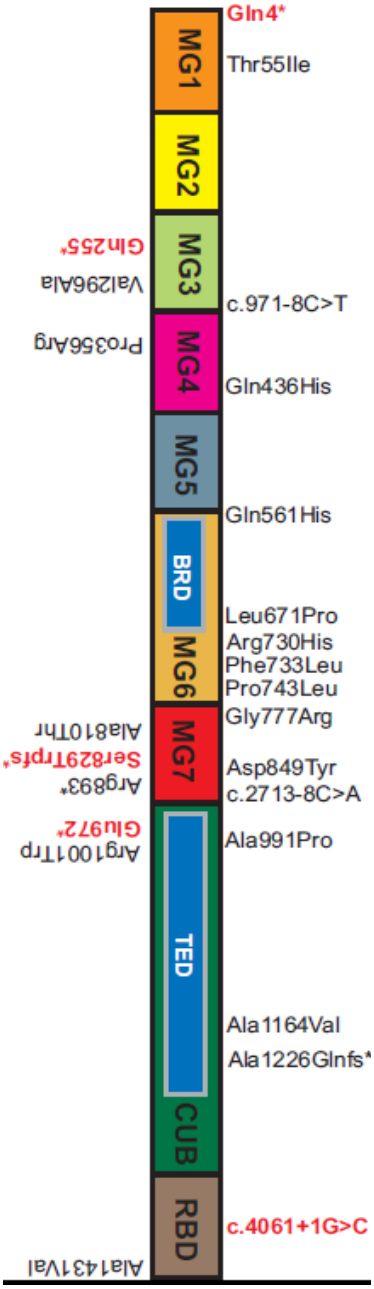

Fig. 2. RNA counts from indigenous Filipinos for [A] *A2ML1*, [B] *RND3* and [C] *AHNAK*. *RND3* and *AHNAK* are upregulated in *A2ML1*-variant carriers (p=0.05) and high *A2ML1*-expressors (*RND3* 2.7-log2foldΔ, adj-p=4.1x10^{-6}; *AHNAK* 1.5-log2foldΔ, adj-p=0.003).

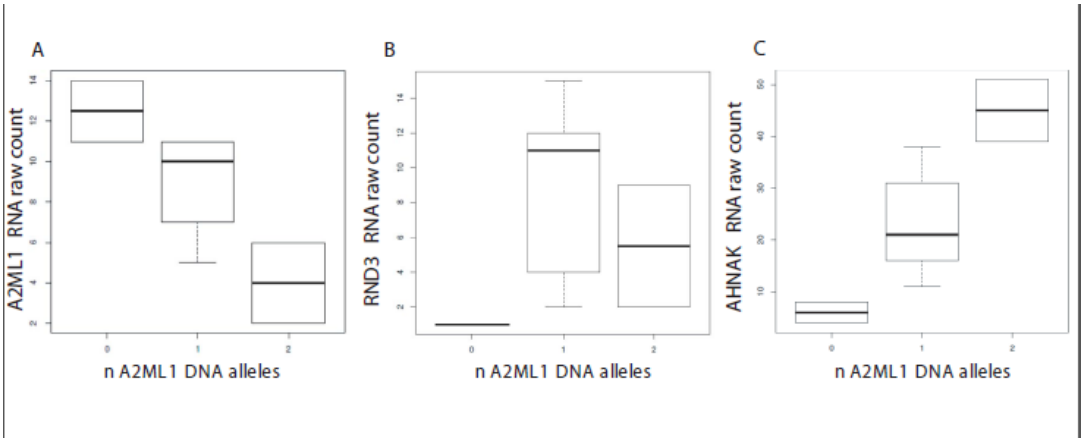

Fig. 3. Heatmap for top 20 differentially expressed genes (plus *RND3* and *AHNAK*) according to *A2ML1* expression in 23 otitis media patients from Colorado. For each data point, the row mean has been subtracted.